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Structure-Cytotoxic Activity Relationships of Simple Hydroxylated Coumarins

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Abstract. *Several hydroxylated and/or methoxylated coumarin derivatives were tested for their relative cytotoxicity on four human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, melanoma A-375 and promyelocytic HL-60) and three normal human cells (gingival fibroblast HGF, periodontal ligament fibroblast HPLF and pulp cell HPC). Tumor cell-specific cytotoxicity was detected in all 6,7-dihydroxy-substituted coumarins only. The observations indicate that the tumor-specific cytotoxicity of the naturally occurring coumarin esculetin can be further enhanced by proper substitutions at 3- and/or 4-position(s) of the molecule. Agarose gel electrophoresis revealed that esculetin and its derivatives with tumor-specific cytotoxicity induce internucleosomal DNA fragmentation in HL-60 cells.*

Coumarins are widely distributed in nature and exhibit a broad pharmacological profile, including anticancer activity (1). However, coumarin, 4- and 7- hydroxycoumarin when tested against P-815 and P-388 tumor cells *in vitro* showed no antitumor potency (2). A later report suggested that coumarin and 7-hydroxycoumarin inhibit the growth of tumors that have reached a certain size but did not prevent the formation of tumors after exposure to the carcinogen (3). They have also been shown to stimulate apoptosis in HL-60 cells and have direct antitumor (cytostatic) activity (4). There are also reports of antitumor effects of other natural and synthetic coumarins (5-12). Various coumarins, especially those possessing ortho-dihydroxyl functions, are capable of scavanging superoxide anions generated by activated phagocytic neutrophils (13) and displayed marked cytotoxic effects (14). Esculetin (6,7-dihydroxycoumarin) is known to induce apoptosis in human leukemia cells by increasing the cytosolic translocation of cytochrome c and activation of cysteine protease 32 kDa proenzyme (15). However, few efforts (14) have been made to establish the relationship between the structure and cytotoxic activity of coumarins in genaral or esculetin in particular. In order to gain insight into the mode of cytotoxic action of coumarin derivatives and to apply this knowledge for the development of more potent cytotoxic coumarin derivatives, we have screened some hydroxylated coumarins for their effects on four human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, melanoma A-375, and promyelocytic leukemia HL-60) and three normal human cells (gingival fibroblast HGF, periodontal ligament fibroblast HPLF and pulp cell HPC).

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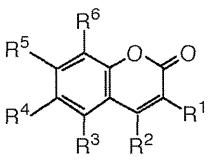
Materials and Methods

Chemicals. The following chemicals were obtained from Tokyo Kasei Co., Tokyo, Japan: coumarin (**C1**) (C0395), 7-hydroxycoumarin (**C2**) (H0236), 6,7-dihydroxycoumarin (**C3**) (E0386), 7-hydroxy-6-methoxycoumarin (**C4**) (S367), 7-hydroxy-4-methylcoumarin (**C6**) (M0453), 6-hydroxy-4-methylcoumarin (**C7**) (H1005), 6,7-dihydroxy-4-methylcoumarin (**C8**) (M0766), 7-methoxycoumarin (**C12**) (M1393), 6-methoxycoumarin (**C13**) (M1398), gallic acid (**C24**) (G0011), caffeic acid (**C25**) (C0002). The remaining chemicals were supplied as indicated: Dulbecco's modified Eagle medium (DMEM), RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Biosci., Lenexa, KS, USA); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chem. Ind., St. Louis, MO, USA).

The synthesis of other coumarins (**C5, C9-11** and **C14-C23**) tested will be reported elsewhere (16).

Cell culture. Human promyelocytic leukemia (HL-60) cells were maintained at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA) in a humidified 5% CO_2 atmosphere. Human oral squamous cell carcinoma (HSC-2) cells were maintained as a



C1-23

	R^1	R^2	R^3	R^4	R^5	R^6
C1	H	H	H	H	H	H
C2	H	H	H	H	OH	H
C3	H	H	H	OH	OH	H
C4	H	H	H	OCH_3	OH	H
C5	H	H	OCH_3	OCH_3	OH	H
C6	H	CH_3	H	H	OH	H
C7	H	CH_3	H	OH	H	H
C8	H	CH_3	H	OH	OH	H
C9	H	CH_3	OH	H	OH	H
C10	H	CH_3	H	OH	OCH_3	H
C11	H	CH_3	H	OCH_3	OH	H
C12	H	CH_3	H	H	OCH_3	H
C13	H	CH_3	H	OCH_3	H	H
C14	CH_3	H	H	H	OH	H
C15	CH_3	CH_3	H	H	OH	H
C16	CH_3	CH_3	H	OH	OH	H
C17	CH_3	CH_3	H	OH	OCH_3	H
C18	CH_3	CH_3	H	OCH_3	OH	H
C19	CH_3	CH_3	H	H	OCH_3	OH
C20	$-(CH_2)_3-$		H	OH	OH	H
C21	$-(CH_2)_3-$		OH	H	OH	H
C22	$-(CH_2)_3-$		H	OH	OCH_3	H
C23	$-(CH_2)_3-$		H	OCH_3	OH	H

Figure 1. *Structures of coumarins studied.*

monolayer culture at 37°C in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL) supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere and subcultured by trypsinization. Human gingival fibroblasts (HGF) were isolated from healthy gingival biopsies of a 10-year-old female, as described previously (17). Cells between the fifth and seventh passages were used.

Cytotoxic activity. Cells were incubated for 24 hours with graded concentrations of test samples in culture medium. Except in the case of HL-60 cells, the viable cell numbers were determined by MTT method (17). Trypan blue exclusion method was used in experiments with HL-60 cells.

Assay for DNA fragmentation. HL-60 cells were lysed with 50 μL lysis buffer [50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsarcosinate]. The solution was incubated sequentially with 4 mg/mL RNase A for 60 minutes at 50°C, and 4 mg/mL proteinase K for 60 minutes at 50°C. DNA was extracted and precipitated by ethanol, then dissolved in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0). DNA, equivalent to 5 x 10^5 cells, was applied to 1.8% agarose gel electrophoresis. The DNA fragmentation pattern was examined in photographs taken under UV illumination (18).

Results and Discussion

The structures of the coumarin derivatives screened for their tumor-specific cytotoxic potentials are shown in Figure 1. Their estimated 50% cytotoxic concentrations (CC_{50} values) for the four tumor cell lines and three other normal cells are summarized in Table I. Cytotoxicity data for two naturally occuring phenolic acids (gallic and caffeic acids) with certain structural analogies with the hydroxylated coumarins tested are also included in this table.

In concentrations up to 200 μg/mL, no or only minimal cytotoxicity of most coumarin derivatives could be detected in normal cells. On the contrary, however, concentration-dependent cytotoxicity for several of them were observed in tumor cell lines. Data summarized in Table I reveal that the CC_{50} values for many of them for diverse tumor cell lines were less than 100 μg/ml. Such was specially the case for the four 6,7-dihydroxycoumarin derivatives **C3, C8, C16** and **C20.** Thus, although coumarin itself (**C1**) and its 7-hydroxy- (**C2**), 6-methoxy-7-hydroxy (**C4**) and 5,6-dimethoxy- (**C5**) derivatives were relatively non-toxic to all cell lines used, its 6,7-dihydroxy derivative (**C3;** esculetin) revealed a tumor cell line-specific cytotoxicity with a selectivity index (SI)>5.1. These observations are in agreement with the reports that the secondary plant metabolite esculetin (**C3**) is cytotoxic for human leukemia (15) and other tumor cell lines (14) and that it is relatively non-toxic to normal human cells (13). In addition, they define the minimal substitution pattern in the coumarin molecule necessary for obtaining cancer cell-specific cytotoxic agents.

Amongst the eight 4-methylcoumarin derivatives (**C6-C13**) tested, the most potent and tumor-specific cytotoxicity was observed in the 6,7-dihydroxylated molecule (**C8**) only. Analogous was also the situation for the 3,4-dimethyl- (**C15-C19**) or 3,4-cycloalkyl- (**C20-C23**) substituted coumarin derivatives. Although the SI values for all the four *ortho*-dihydroxycoumarins tested were >5, such was not the case for *ortho*-dihydroxylated reference molecule caffeic acid (or for the trihydroxylated molecule gallic acid). These observations strongly suggest that 6,7-dihydroxycoumarin represents a specific pharmacophore moiety suitable for designing tumor-specific cytotoxic agents.

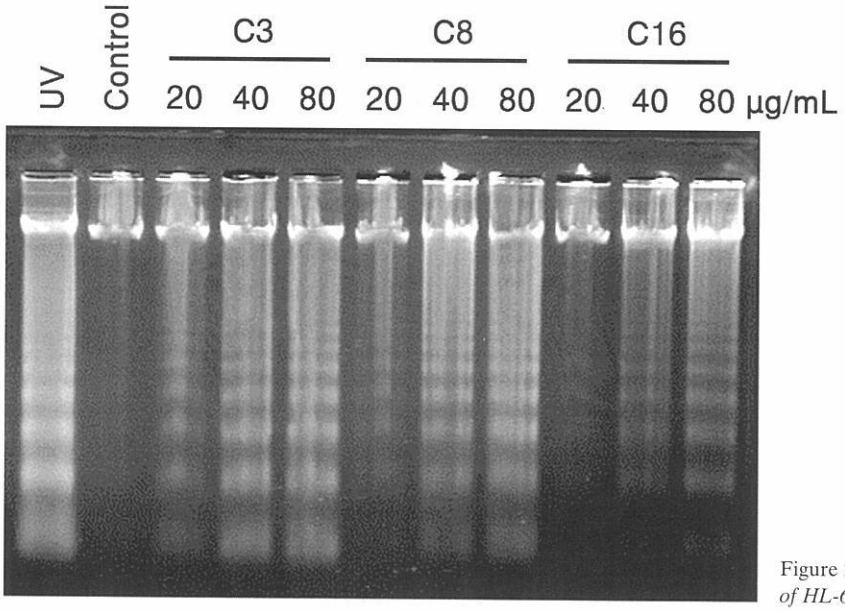


Figure 2. *Induction of internucleosomal DNA fragmentation of HL-60 cells by 6,7-dihydroxycoumarins* (C3, 8 *and* 16).

The mean SI values of 6,7-dihydroxycoumarin (**C3**) was less than that of its 4-methyl (**C8**) or 3,4-dimethyl (**C16**) or 3,4-cycloalkyl (**C20**) derivatives. Since theoretically the alkyl-substituted molecules should be more lipophilic than the parent molecule, their observed higher efficacy could as well be due to their better cellular bio-availability. If such were indeed the case, it should be possible to design tumor-specific cytotoxic agents, more potent than esculetin, by proper substitutions in the 3 and/or 4 positions of the molecule. However, for such purposes it is essential to establish that the cytotoxic mechanisms of the derivatives are similar to that of the parent molecule esculetin (**C3**).

It has been reported (15) that induction of apoptosis by esculetin (**C3**) is involved in its cytotoxic effects on HL-60 cells. It was of interest, therefore, to check as to whether its alkylated derivatives could also induce DNA fragmentation in these cells. The results of an initial experiment shown in Figure 2 reveal that such is indeed the case. It seems reasonable, therefore, that a more potent or better bioavailable esculetin-like and cancer cell line-specific cytotoxic agent is an achievable goal. Synthesis and/or search for naturally occurring 6,7-dihydroxycoumarins, with appropriate 3- and/or 4-substitution patterns from plants and other natural sources, seems to be a suitable means for achieving such goals.

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Table I. Cytotoxic activity of coumarins against tumor and normal cells.

Compound	50% cytotoxic concentration (CC_{50}, μg/mL)							SI[a] = CC_{50} (normal)/ CC_{50} (tumor)
	Human tumor cell lines				Normal cells			
	HSC-2	HSC-3	A-375	HL-60	HGF	HPLF	HPC	
C1	>200	>200	>200	>200	>200	>200	>200	-
C2	166	>200	>200	142	>200	>200	>200	><1.1
C3	42	32	70	13	>200	>200	>200	>5.1
C4	>200	>200	>200	>200	>200	>200	>200	-
C5	>200	>200	>200	>200	>200	>200	195	-
C6	168	176	>200	125	>200	>200	183	><1.2
C7	124	192	177	123	>200	>200	117	>1.1
C8	33	16	34	13	>200	>200	>200	>8.3
C9	80	>200	95	49	>200	>200	>200	><1.9
C10	>200	>200	196	>200	>200	>200	>200	-
C11	156	>200	177	>200	>200	>200	>200	-
C12	40	>200	>200	>200	>200	>200	>200	-
C13	62	>200	>200	>200	>200	>200	>200	-
C14	78	157	148	133	>200	>200	168	>1.5
C15	77	128	91	150	141	183	161	1.5
C16	19	9	33	18	200	>200	152	>9.3
C17	33	46	75	>200	>200	200	182	-
C18	120	>200	>200	>200	>200	>200	>200	-
C19	39	70	106	>200	>200	>200	133	-
C20	23	19	22	9	>200	>200	>200	>11.0
C21	81	134	69	47	168	164	138	>1.9
C22	17	160	>200	>200	>200	>200	>200	-
C23	19	181	>200	>200	>200	>200	177	-
Gallic acid	20	17	55	16	38	48	69	1.9
Caffeic acid	>200	156	>200	159	158	166	>200	-
A_{540}	1.011	0.316	0.540	-	0.303	0.323	0.285	

Near confluent cells were incubated for 24 hours without or with various concentrations of each compound, and the relative viable cell number (absorbance at 540 nm of the MTT-stained cell lysate) was determined by the MTT method. The viable cell number of HL-60 cells was determined by trypan blue exclusion. The CC_{50} was determined from the dose-response curve. Each value represents the mean from duplicate determinations.
[a]determined by the equation: $SI=[(CC_{50})HGF + (CC_{50})HPLF + (CC_{50})HPC / (CC_{50})HSC\text{-}2 + (CC_{50})HSC\text{-}3 + (CC_{50})A\text{-}375 + (CC_{50})HL\text{-}60] \times 4/3$

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